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Effects of Structure on Binding to the 2,3,7,8-TCDD Receptor Protein and AHH Induction—Halogenated Biphenyls

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The quantitative structure–activity relationships (QSARs) for polychlorinated biphenyl (PCB) congeners have been determined by comparing the EC_{50} values for three *in vitro* test systems, namely, aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin *O*-deethylase (EROD) induction in rat hepatoma H-4-II-E cells and competitive binding avidities to the rat cytosolic receptor protein (using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin as a radioligand). For several PCB congeners that are *in vivo* inducers of rat hepatic microsomal AHH, there was a linear correlation between the $-\log EC_{50}$ values for receptor and the $-\log EC_{50}$ values for AHH (or EROD) induction; moreover, a comparable linear relationship was observed between the $-\log EC_{50}$ values for AHH and EROD induction. Previous *in vivo* studies have shown that the most active PCB congeners 3,3',4,4'-tetra-, 3,4,4',5-tetra-, 3,3',4,4',5-penta-, and 3,3',4,4',5,5'-hexachlorobiphenyl, cause many of the biologic and toxic effects reported for the highly toxic halogenated aryl hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Moreover, the mono*ortho*-substituted homologs of the four coplanar PCBs also elicit comparable *in vivo* biologic and toxic responses. It was evident from the QSARs for PCBs that there was an excellent correspondence between the *in vivo* and *in vitro* potencies of the individual PCB congeners.

The effects of substituents on both receptor binding and AHH/EROD induction was determined for a series of 4'-substituted (X)-2,3,4,5-tetrachlorobiphenyls (where X = H, Cl, Br, I, OH, OCH_3, NO_2, $COCH_3$, F, CF_3, CH_3, C_2H_5, *i*-C_3H_7, *n*-C_4H_9 and *t*-C_4H_9). Not unexpectedly, there was a linear relationship between the $-\log EC_{50}$ values for AHH and EROD induction, and these results confirm that both enzymatic oxidations are catalyzed by the same cytochrome P-450 isozyme(s). The effects of substituent structure on receptor binding for 12 substituents was subjected to multiple regression analysis which correlates the relative binding affinities of the compounds with the physical chemical characteristics of the substituents. The analysis gave the following equation: $\log (1/EC_{50}) = 1.53\sigma + 1.47\pi + 1.09\ HB + 4.08$ for $n = 12$, $s = 0.18$, $r = 0.978$; where n is the number of substituents, s is the standard deviation, r is the correlation coefficient, and σ = electronegativity, π = hydrophobicity (log P) and HB = hydrogen bonding capacity for the substituent groups. The data suggest that the latter three parameters facilitate the interaction between the ligand and the cytosolic receptor protein. The effects of two lateral substituents on ligand–receptor interactions are not readily explained by the above relationship and may depend on other substituent physical chemical parameters.

Introduction

Pharmacogenetic studies have played an important role in understanding the biologic and toxic effects of carcinogens, drugs and related xenobiotics. Nebert and co-workers have investigated the toxic, carcinogenic, and biologic activities of polynuclear aromatic hydrocarbons (PAHs) in genetically inbred mice strains, and their results have suggested a mechanism of action for these compounds (*1–4*). The activity of 3-methylcholanthrene (3-MC) and several related PAHs as inducers of hepatic and extrahepatic drug-metabolizing enzymes is remarkably dependent on the strain of mice used. Hepatic microsomal aryl hydrocarbon hydroxylase (AHH), a cytochrome P-448-dependent monooxygenase, is readily induced by 3-MC in several strains of

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mice, typified by the "responsive" C57BL/6J strain; in contrast, 3-MC does not induce AHH in nonresponsive strains, typified by the DBA/2J mice (*5,6*). Moreover, responsive mice are more susceptible than nonresponsive mice to the toxic (inflammation, fetotoxicity, primordial oocyte depletion) and carcinogenic effects of PAHs at organs/tissues in direct contact with the applied PAHs (*1–13*). The gene complex responsible for mediating the effects of PAHs has been designated the *Ah* locus which may comprise regulatory, structural, and possibly temporal genes. Extensive studies on genetically inbred responsive and nonresponsive mice (and their backcrosses) suggests that the differences in susceptibility are related to the levels of the *Ah* regulatory gene product, the cytosolic *Ah* receptor protein. The receptor protein interacts with the PAH ligands and the resultant PAH:receptor ligand complex translocates into the nucleus and presumably initiates the toxic and biologic effects via a process comparable to that proposed for the steroid hormones (*1–6*).

Genetic, biochemical and toxicologic studies also support the role of the *Ah* receptor protein in mediating the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*14–17*). For example, competitive binding studies with radiolabeled and unlabeled 2,3,7,8-TCDD and 3-MC confirms that both compounds bind to the same receptor protein (*18*). The murine *Ah* receptor concentrations in C57BL/6J mice vary from 45 to 75 fmole/mg cytosolic protein, whereas this protein is not detected in the cytosol of DBA/2J mice (note: nuclear receptor levels have been detected in this mouse strain) (*18–20*). 2,3,7,8-TCDD induces AHH and causes cleft palate, a wasting syndrome, immunotoxicity and thymic atrophy in responsive C57BL/6J mice; some of these effects are observed in the nonresponsive DBA/2J mice but only at relatively high dose levels (*14–17, 21–23*). Backcross experiments with DBA/2J and C57BL/6J mice give results consistent with the segregation of the activity of 2,3,7,8-TCDD with the *Ah* locus (*16,17,21,23,25,26*). There are marked effects of structure on the activity of polychlorinated dibenzo-*p*-dioxins (PCDDs); the most active compound, 2,3,7,8-TCDD, is substituted at all four lateral positions. Moreover, the addition of Cl groups or the removal of lateral substituents tends to reduce the activity of the resultant congeners (*17,21,22,26–29*). Several halogenated aromatic compounds, such as 2,3,7,8-tetrachlorodibenzofuran (TCDF), 3,3',4,4',5,5'-hexabromo- and hexachlorobiphenyl, which are approximate isostereomers of 2,3,7,8-TCDD, exhibit similar toxic and biologic activities (*17,21,29–37*).

The results obtained for the toxic halogenated aromatics show a correlation between the toxicity of an individual compound, its receptor binding avidity, and potency as an inducer of AHH. These results support the role of the *Ah* receptor in mediating many of the toxic and biologic responses elicited by this class of chemicals.

Polychlorinated Biphenyls (PCBs) and Polybrominated Biphenyls (PBBs)—QSAR

PCBs

In vivo structure–activity relationships developed for PCB congeners show that the most active compounds, 3,3',4,4'-tetra-, 3,3',4,4',5-penta-, and 3,3',4,4',5,5'-hexachlorobiphenyl, elicit toxic and biologic effects which are comparable to those observed after treatment with 2,3,7,8-TCDD. These compounds are potent inducers of AHH and ethoxyresorufin *O*-deethylase (EROD) in several mammalian species and induce cytochromes P-450c and P-450d in male Long-Evans rats (*38–43*). Although extensive toxicity studies have not been reported for all three congeners, it is apparent that one or more of these PCBs elicit the following toxic effects; a wasting syndrome, thymic atrophy, hepatic damage, reproductive toxicity, differential effects on genetically inbred C57BL/6J and DBA/2J mice, porphyria, immunotoxicity, and dermal toxicity (*42,44–51*).

Studies in our laboratory have shown that all the mono-*ortho* analogs of the 3,3',4,4'-tetra-, 3,4,4',5-tetra-, 3,3',4,4',5-penta- and 3,3,4,4,5,5'-hexachlorobiphenyls also induce hepatic microsomal AHH and EROD in male Wistar rats and C57BL/6J mice. However, in contrast to the highly toxic coplanar PCBs, these compounds resemble phenobarbitone (PB) plus 3-MC (coadministered) as inducers of hepatic microsomal drug-metabolizing enzymes (*42,52,53*). Despite this apparent bifunctional or "mixed-type" enzyme induction activity, there is evidence in the literature that many of these compounds also cause toxic effects similar to those observed after treatment with 2,3,7,8-TCDD. For example: 2,3',4,4',5-penta-, 2,3,3',4,4'-penta-, 2,3,3',4,4',5-hexa-, and 2,3,3',4,4',5'-hexachlorobiphenyl cause thymic atrophy in rats (*50*). 2,3,3',4,4'-Pentachlorobiphenyl administered to mice and rats results in a wasting syndrome (weight loss), edema, liver lipid accumulation, extensive hepatic damage, and splenic atrophy (*54*); 2,3',4,4',5-pentachlorobiphenyl causes 100% embryo mortality in eggs from pullets receiving the PCB in their diet at a level of 20 ppm (*55*); administration of 2,3',4,4',5-penta- and 2,3,3',4,4',5-hexachlorobiphenyl to rats caused increased liver weights, increased liver lipids and thymic atrophy (*56*). These data indicate that at least five of the mono-*ortho* analogs of the coplanar PCBs elicit toxic effects that qualitatively resemble those observed after treatment with 2,3,7,8-TCDD.

The *in vivo* structure–activity relationships (SARs) provide qualitative support for the proposed receptor-mediated hypothesis for the mechanism of action of 2,3,7,8-TCDD and related toxic halogenated aryl hydrocarbons. Quantitative structure–activity relation-

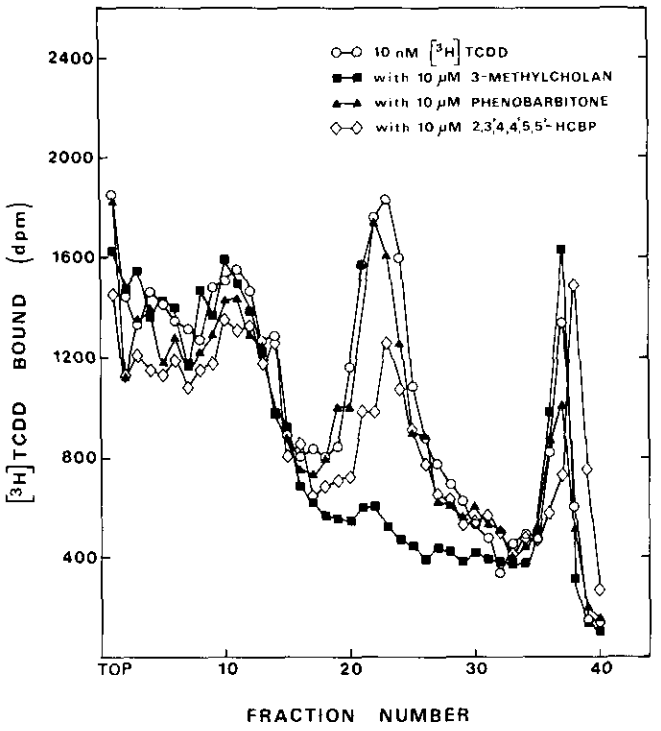

FIGURE 1. Sucrose density gradient analysis after incubation of 10 nM [^{3}H]-2,3,7,8-TCDD with the rat cytosolic receptor protein and no competitor; 10 μM 2,3,7,8-TCDD, 10 μM 3-MC, and 10 μM PB.

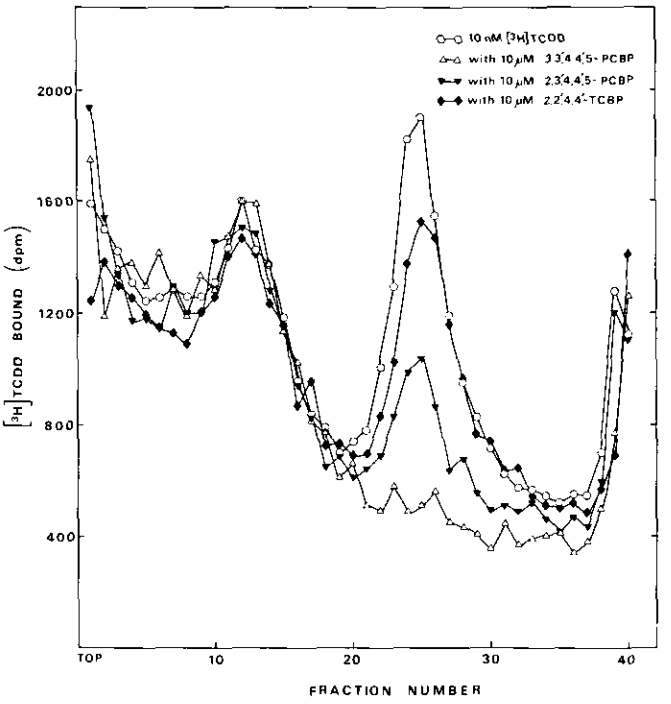

FIGURE 2. Sucrose density gradient analysis after incubation with 10 nM [^{3}H]-2,3,7,8-TCDD with the rat cytosolic receptor protein and 10 μM 3,3',4,4',5'-penta-, 2,3,3',4,4'-penta-, and 2,2',4,4'-tetrachlorobiphenyl.

ships (QSARs) for the PCBs have been obtained by using interrelated bioassays systems, namely, the competitive binding affinities of PCBs for the 2,3,7,8-TCDD hepatic cytosolic receptor protein from male Wistar rats (*57*) and the AHH and EROD induction potencies of these compounds in rat hepatoma H-4-II-E cells in culture (*34*). The former assay measures the important interaction between the toxin or ligand and the receptor protein, whereas the induction assay measures one of the biologic consequences (i. e., the induction of cytochrome P-448-dependent monooxygenases of this initial interaction). Dose–response studies are also readily determined by using *in vitro* assays, and the pharmacokinetic and metabolic factors which play a role in *in vivo* studies are minimized.

The QSAR for the binding avidities of PCBs to the cytosolic receptor protein have been reported by using the sucrose density gradient assay system (*57*). Figure 1 illustrates the [^{3}H]-2,3,7,8-TCDD-receptor protein binding peak which sedimented at approximately fraction 25 and corresponded to a sedimentation rate of 8–10S (dependent on ionic concentration of the buffer). Competition experiments with 10 μM nonradiolabeled 2,3,7,8-TCDD or 3-MC completed eliminated this peak, whereas competition experiments with PB did not significantly decrease the area of this radioactive binding peak. Figure 2 illustrates the sucrose density gradient profiles observed after competive binding experiments of the cytosolic receptor protein–[^{3}H]-2,3,7,8-TCDD complex with 10 μM 3,3',4,4',5-pentachlorobiphenyl and 2,3',4,4',5- and 2,2',4,4'-tetrachlorobiphenyl. The highly toxic 3-MC-type inducer, 3,3',4,4',5-pentachlorobiphenyl, completely eliminates the radiolabeled binding peak, whereas the less toxic mixed-type inducer only partially reduces the area of this peak. Although 2,2',4,4'-tetrachlorobiphenyl is a relatively nontoxic PCB congener that strictly resembles PB in its mode of induction of the hepatic microsomal monooxygenases, this compound does competitively displace some of the radiolabeled 2,3,7,8-TCDD. Dose–response-competition experiments with several other PCB congeners that do not induce AHH (i.e., 2,3,4,5-tetra- and 2,2',4,4',5,5'-hexachlorobiphenyl) showed that at high concentrations there is some competition with [^{3}H]-2,3,7,8-TCDD for the receptor binding protein. The biological significance of this weak binding has not been determined.

Table 1 summarizes the binding avidities of 14 PCBs, and the data complement the results illustrated in Figure 1; the coplanar PCBs are the most avid competitors for binding to the receptor protein; the mono*ortho*-substituted analogs of the coplanar PCBs also bind to the receptor but with lower affinities than the coplanar compounds. These *in vitro* data parallel the *in vivo* SAR for PCBs and confirm that the most active compounds are substituted in both *para* and two or more *meta* positions and that the introduction of a single *ortho* sub-

Table 1. PCBs: a comparison of the effects of structure on their cytosolic receptor binding avidities and AHH/EROD induction potencies.

PCB congener	No.	Receptor binding	–log EC_{50} AHH induction	–log EC_{50} EROD induction
3,3',4,4'5-Penta	1	6.89	9.62	9.61
3,3',4,4'-Tetra	2	6.15	7.55	7.05
2,3,4,4',5-Penta	3	5.39	6.02	6.25
2,3,3',4,4'-Penta	4	5.37	7.06	6.92
2,3,3',4,4',5'-Hexa	5	5.33	6.15	5.90
Aroclor 1254		5.22	5.42	5.34
2,3,3',4,4',5-Hexa	6	5.15	5.68	6.05
2,3',4,4',5-Penta	7	5.04	4.94	5.05
2',3,4,4',5-Penta	8	4.85	5.41	5.95
2,3',4,4',5,5'-Hexa	9	4.80	4.88	5.05
2,3,4,4'-Tetra	10	4.55	4.96	5.72
2,2',4,4',5,5'-Hexa	11	4.10	—	—
2,3',4,4',5', 6-Hexa	12	4.00	—	—
2,2',4,4'-Tetra	13	3.89	—	—
2,3,4,5-Tetra	14	3.85	—	—
PB		—	—	—
3-MC		7.60	5.89	6.20
TCDD		8.00	10.02	10.10

stituent into the biphenyl ring diminishes but does not eliminate the activity of the resultant compounds.

Table 1 also summarizes the effects of structure on the activity of PCBs as inducers of AHH and EROD in rat hepatoma H-4-II-E cells in culture. A plot of receptor binding affinities versus AHH or EROD induction potencies (Figs. 3 and 4) illustrates the linear correlation between these two biologic parameters and thus supports the role of the receptor in mediating the induction of the cytochrome P-448-dependent monooxygenases.

PBBs

In vivo studies with purified PBB congeners indicate that their biologic and toxic properties and the effects of structure on activity are comparable to those observed for the PCBs. The coplanar PBBs substituted in both *para* and two or more *meta* positions are highly toxic (*29,42,58–61*), induce microsomal AHH and the associated cytochrome P-450 isozymes in rats (*42,60,62*), and elicit differential biologic and toxic effects in genetically inbred C57BL/6J and DBA/2J mice (*50*). Moreover, many of the mono*ortho* analogs of the coplanar PBBs are mixed-type inducers (i.e., inducers of cytochromes P-450a–P-450e) (*42,60,62–65*) and also cause some of the toxic effects observed for the coplanar congeners (*42,63–66*). A detailed *in vitro* QSAR analysis has not been reported for PBB congeners; however, both 3,3',4,4',5,5'-hexa- and 3,3',4,4'-tetrabromobiphenyl competitively displace radiolabeled 2,3,7,8-TCDD from the cytosolic receptor protein (*29,58*). Figure 5 illustrates the sucrose density gradient analysis obtained after incubation of [^{3}H]-2,3,7,8-TCDD with rat hepatic cytosol and selected PBB congeners. Like the PCBs, the coplanar 3,3',4,4',5-pentabromobiphenyl completely displaces the radiolabel, the 2,3,3',4,4',5'-hexabromobiphenyl partially displaced the radiolabel whereas 2,2',5,5'-tetrabromobiphenyl, a relatively nontoxic congener that does not induce AHH, was the least active competitor. These results were similar to those reported for the PCBs.

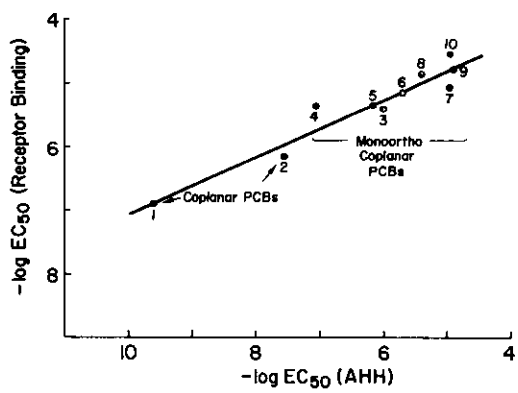

FIGURE 3. Plot of the $-\log EC_{50}$ values of the receptor binding avidities vs. AHH induction potencies for a series of PCB isomers and congeners (Table 1).

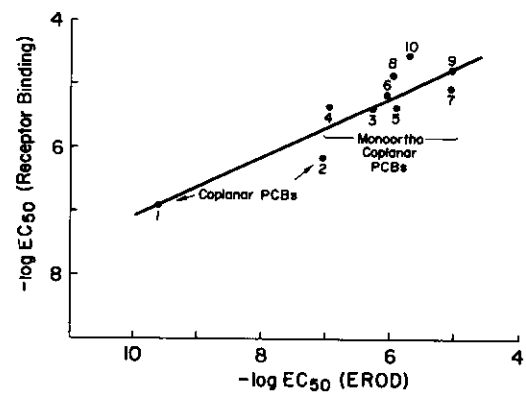

FIGURE 4. Plot of the $-\log EC_{50}$ values of the receptor binding avidities vs. EROD induction potencies for a series of PCB isomers and congeners (Table 1).

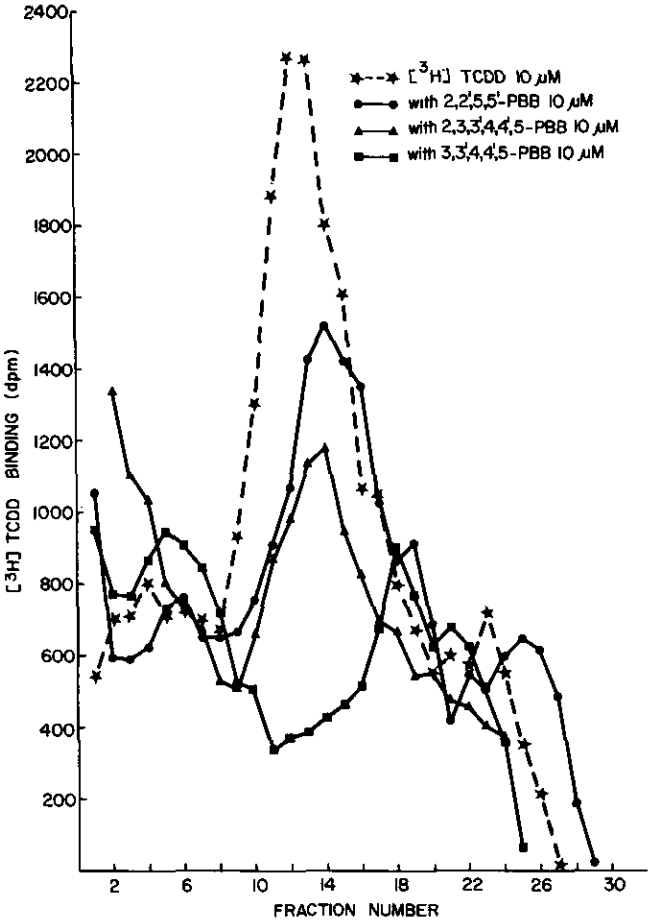

FIGURE 5. Sucrose density gradient analysis after incubation with 10 nM [^{3}H]-2,3,7,8-TCDD and 10 μRM 3,3',4,4',5-penta-, 2,3,3',4,4',5'-hexa-, and 2,2'5,5'-tetrabromobiphenyl.

Andres and co-workers (*61*) recently compared the biologic and toxic effects of a series of 3,3',4,4'-tetrahalobiphenyls that differed in their Br/Cl ratios. It was apparent that for the *in vivo* studies the most active and least active compounds were the 3,3',4,4'-tetrabromo- and 3,3',4,4'-tetrachlorobiphenyls, respectively. The effects of Br versus Cl were more dramatically illustrated by the comparison of *in vitro* receptor binding affinities and AHH/EROD induction potencies in rat hepatoma H-4-II-E cells in culture (Fig. 6). The QSAR studies clearly illustrate a linear relationship between the binding and induction parameters and support the relationship between these two effects. It is also evident that for the 3,3',4,4'-tetrahalobiphenyls, bromo substituents facilitate ligand–receptor binding more than chloro groups. The reason for these substituent effects are discussed in the following section.

Substituted Halogenated Biphenyls

The *in vivo* and *in vitro* studies with structurally diverse PCBs and PBBs support a common receptor-mediated mechanism of action for halogenated biphenyls, PCDDs and PCDFs. Despite the critical role played by the *Ah* receptor protein in the biochemistry and toxicology of halogenated aromatics and PAHs, this protein has not been purified from any species. However, the nature of the protein binding sites for halogenated aromatics have been studied by several groups, and their results indicate the following.

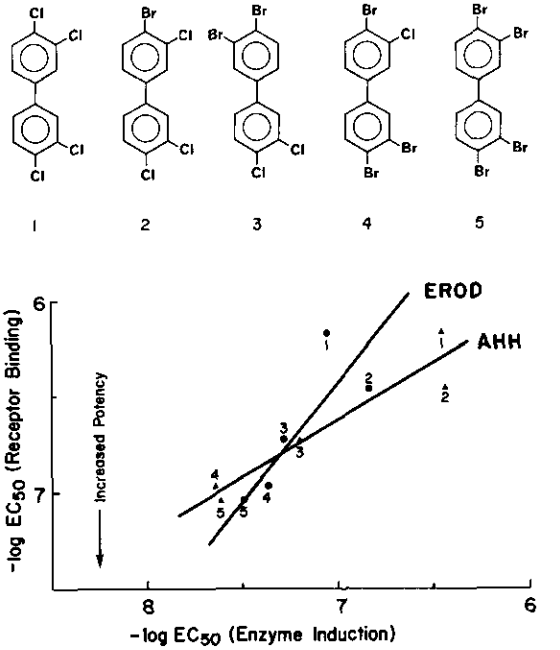

FIGURE 6. Plot of the $-\log EC_{50}$ values of the receptor binding avidities vs. AHH and EROD induction potencies for a series of 3,3',4,4'-tetrahalobiphenyls.

Based on the high binding affinity of 2,3,7,8-TCDD, a ligand should be relatively flat or planar, and deviations from this structural feature (e.g., mono*ortho*-substituted halogenated biphenyls) result in diminished binding affinities (*21,26,36*) of the ligands. The optimal two-dimensional shape of a ligand corresponds to a 3 × 10 Å rectangle (*17,21,37*). Maximum binding affinity requires the substitution of Cl at all four lateral positions for the PCDDs and PCDFs or at four to six lateral positions for PCBs and PBBs (*17,21,37,57*). Bromo-substituted biphenyls and PCDDs exhibit higher binding affinities than their chlorinated analogs (*61,67,68*). Polarizability and the electron-acceptor properties of the lateral substituents are proposed to be important properties which facilitate binding to the receptor (*69–71*).

Substituent effects on the activity of chemicals are routinely determined to facilitate the design of active drugs and agricultural chemicals. This approach can also be used to study the effects of substituents on the interaction of halogenated aromatics with the 2,3,7,8-TCDD receptor protein. The model compounds chosen for this study were a series of 4'-substituted-2,3,4,5-tetrachlorobiphenyls. The PCB member of this series, 2,3,4,4',5-pentachlorobiphenyl, is a mixed-type inducer of microsomal monooxygenases and exhibits many of the properties characteristic of 2,3,7,8-TCDD and re-

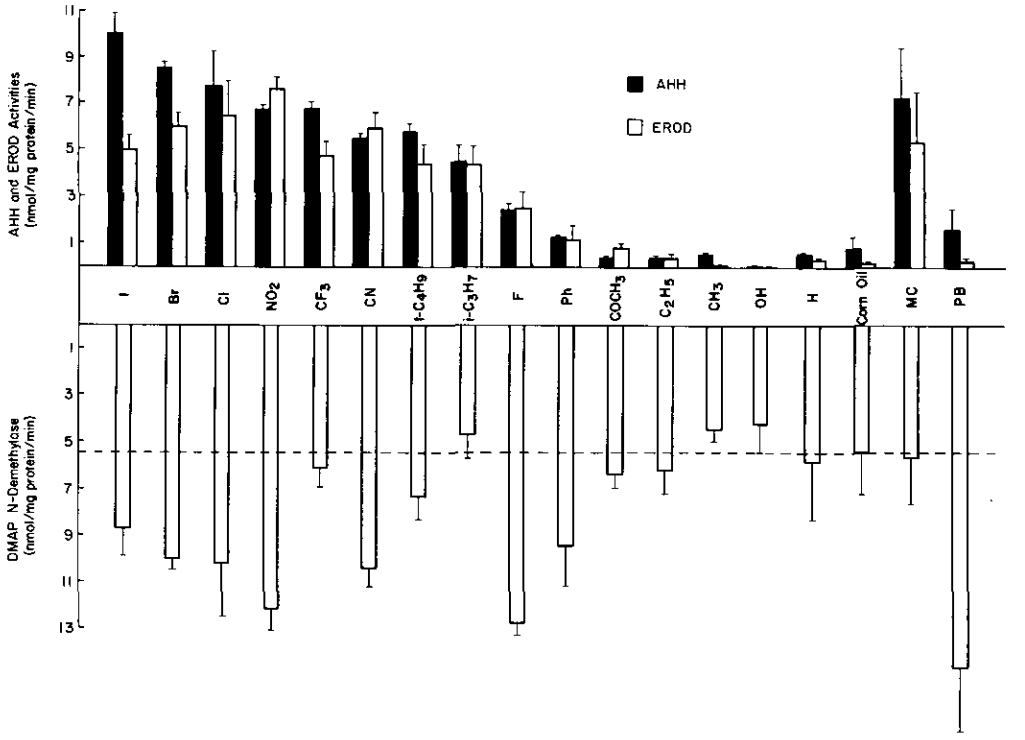

FIGURE 7. *In vivo* AHH, EROD, and *N*-DMAP induction potencies of 4′-substituted 2,3,4,5-tetrachlorobiphenyls (300 μmole/kg) in immature male Wistar rats.

lated toxic halogenated aromatics. 2,3,4,4′,5-Pentachlorobiphenyl (PCBP) induces microsomal AHH and EROD in male Wistar rats (*33*), C57BL/6J mice (*72*), and rat hepatoma H-4-II-E cells in culture (*34*) and induces cytochromes P-450c and P-450d in male Long-Evans Rats (*42*); PCBP also binds to the rat hepatic cytosolic 2,3,7,8-TCDD receptor protein (*57*). In addition, PCBP causes thymic atrophy in rats and C57BL/6J mice but does not induce AHH or cause thymic atrophy in DBA/2J mice (*72*). Substitution at the 4′ lateral position is important for the activity of this group of halogenated biphenyls, since 2,3,4,5-tetrachlorobiphenyl, the unsubstituted homolog (4′ = H), is a poor ligand for the receptor (*57*), does not induce AHH or EROD, and is relatively nontoxic.

The effects of 14 different 4′-substituted-2,3,4,5-tetrachlorobiphenyls as inducers of rat hepatic microsomal monooxygenases are summarized in Figure 7. The *in vivo* activity of these compounds as inducers of microsomal AHH, EROD and DMAP *N*-demethylase were remarkably dependent on the structure of the 4′-substituent. The 4′-iodo-2,3,4,5-tetrachlorobiphenyl was the most potent inducer of AHH and EROD; however, the 4′-bromo-, 4′-chloro-, 4′-trifluoromethyl, 4′-nitro-,4′-cyano-, 4′-*tert*-butyl-, 4′-isopropyl-, 4′-fluoro-, 4′-phenyl-, 2,3,4,5-tetrachlorobiphenyls also significantly induced both cytochrome P-448-dependent monooxygenases. There were also substituent effects on the activity of these compounds as inducers of DMAP *N*-demethylase; the chloro-, bromo-, fluoro-, nitro-, and cyano-substituted PCBs were inducers, whereas the 4′-trifluoromethyl-, 4′-iodo-, 4′-isopropyl-, and 4′-*tert*-butyl-2,3,4,5-tetrachlorobiphenyls were inactive. A second group of 4′-substituted 2,3,4,5-tetrachlorobiphenyls, namely the 4′-acetyl-, 4′-methyl-, and 4′-hydroxy-2,3,4,5-tetrachlorobiphenyls and 2,3,4,5-tetrachlorobiphenyl do not induce any of the cytochrome P-450-dependent monooxygenases. The rationale for the effects of substituents on the *in vivo* microsomal monooxygenase enzyme-inducing activities of the substituted PCBs is not apparent: One possible explanation for these differences in activity may be associated with hepatic residue levels of these compounds. The results summarized in Table 2 clearly show that the most active AHH and EROD inducers persist in the liver at concentrations which range from 36.34 to 17.04 ppm. However, 4′-isopropyl-, 4′-*tert*-butyl- and 4′-phenyl-2,3,4,5-tetrachlorobiphenyl also significantly induce AHH and EROD and their hepatic levels are in the range of 1 to 4 ppm. Although residue levels may play a role in the *in vivo* potencies of the 4′-substituted-2,3,4,5-tetrachlorobiphenyls, it is probable that many factors (e. g., metabolic rates) including the substituent properties are important determinants for the *in vivo* activities.

The quantitative effects of the 4′-substituents on activity are more readily assessed using the *in vitro* bioassays (*73*) since these procedures minimize the importance of metabolic and pharmacokinetic factors. Table 3 summarizes the -log EC_{50} values for the receptor binding avidities, AHH and EROD induction potencies for thirteen 4′-substituted-2,3,4,5-tetrachlorobiphenyls.

Table 2. Comparative concentrations of 4'-substituted 2,3,4,5-tetrachlorobiphenyls in rat liver.[a]

Treatment	Residue levels, ppm	Number of animals per group, *n*
4'-Iodo-2,3,4,5-tetrachlorobiphenyl	72.08 ± 10.33	3
4'-Bromo-2,3,4,5-tetrachlorobiphenyl	171.04 ± 12.89	3
2,3,4,4',5-Pentachlorobiphenyl	55.53 ± 13.27	4
4'-Fluoro-2,3,4,5-tetrachlorobiphenyl	36.68 ± 1.63	3
4'-Hydro-2,3,4,5-tetrachlorobiphenyl	0.429 ± 0.208	3
4'-Methyl-2,3,4,5-tetrachlorobiphenyl	0.385 ± 0.251	4
4'-Ethyl-2,3,4,5-tetrachlorobiphenyl	3.98 ± 2.94	4
4'-Isopropyl-2,3,4,5-tetrachlorobiphenyl	1.16 ± 0.07	3
4'-Butyl-2,3,4,5-tetrachlorobiphenyl	3.01 ± 0.32	3
4'-Butyl-2,3,4,5-tetrachlorobiphenyl	2.41 ± 1.85[b]	3
4'-Phenyl-2,3,4,5-tetrachlorobiphenyl	3.14 ± 0.07	3
4'-Trifluoromethyl-2,3,4,5-tetrachlorobiphenyl	58.41 ± 7.17	3
4'-Cyano-2,3,4,5-tetrachlorobiphenyl	36.34 ± 1.55	3
4'-Hydroxy-2,3,4,5-tetrachlorobiphenyl	ND[c]	4
4'-Methoxy-2,3,4,5-tetrachlorobiphenyl	2.63 ± 1.40	3
4'-Acetyl-2,3,4,5-tetrachlorobiphenyl	not determined	3
4'-Nitro-2,3,4,5-tetrachlorobiphenyl	101.34 ± 66.63	4
4'-*N*-Acetylamino-2,3,4,5-tetrachlorobiphenyl	not determined	4

[a] Animals were injected intraperitoneally on days 1 and 3 with the 4'-substituted 2,3,4,5-tetrachlorobiphenyls (dissolved in corn oil) at dose levels of 150 μmole/kg per injection and killed on day 6.
[b] Residue levels of this compound in adipose tissue were 325.07 ± 188.36 (n = 4).
[c] ND = non-detectable.

Table 3. 4'-Substituted-2,3,4,5-tetrachlorobiphenyls: effects of substituents on their cytosolic receptor binding avidities and AHH/EROD induction potencies.[a]

4'-Substituted (X)-2,3,4,5-tetrachlorobiphenyls	No.	-log EC_{50}		
		Receptor binding	AHH induction	EROD induction
X = CF_3	1	6.43	8.42	8.62
X = *i*-C_3H_7	2	5.89	6.35	6.74
X = I	3	5.82	7.96	7.68
X = Br	4	5.60	7.52	7.28
X = C_2H_5	5	5.46	5.47	5.31
X = Cl	6	5.33	5.41	5.96
X = CN	7	5.27	4.85	5.05
X = $COCH_3$	8	5.17	5.45	5.43
X = OCH_3	9	4.80	4.59	4.52
X = F	10	4.60	4.57	4.72
X = CH_3	11	4.51	4.82	4.89
X = OH	12	4.05	4.0	4.0
X = H	13	3.85	4.0	4.0

[a] Taken from Bandiera et al. (*73*).

From inspection of the results of competitive binding experiments (Table 3), it is apparent that some substituent groups enhance the binding of 4'-substituted-2,3,4,5-tetrachlorobiphenyls relative to that of 2,3,4,4',5-pentachlorobiphenyl (i. e., 4'-chloro-2,3,4,5-tetrachlorobiphenyl), while others diminished it. The relative competitive potency of the 4'-substituted 2,3,4,5-tetrachlorobiphenyls followed the order $CF_3 > CH(CH_3)_2 > I > Br > CH_2CH_3 > Cl$ for the most effective ligands. In fact, 4'-trifluoromethyl-2,3,4,5-tetrachlorobiphenyl (i. e., 4'-CF_3 derivative) competed with [^{3}H]-2,3,7,8-TCDD for the specific binding sites almost as effectively as 3,3',4,4',5-pentachlorobiphenyl, the most potent competitor of all the PCBs previously tested. The finding that both the 4'-ethyl and 4'-isopropyl compounds were active competitors for the receptor indicates that halogen substitution in both phenyl rings is not a requirement for binding.

Since the ability to bind effectively to the cytosolic receptor is either enhanced or reduced by various substituent groups on the tetrachlorobiphenyl backbone, the variation in biological activity must be dependent on the properties inherent in the substituents. Quantitative analysis of substituent structure–activity effects can be used to study the intermolecular interactions between ligands and their receptors (*74–77*). In general, drug–receptor interactions involve such forces as hydrophobic bonding, hydrogen bonding, van der Waals energy, electrostatic energy, valence-bond energy, and repulsion or strain energies of the bonds (*77*). The

strength of the interaction depends upon the use of these various energies, which for a series of substituted congeners, can be approximated by the use of free-energy-related physiochemical substituent parameters (*74–78*). For the present series of 4′-substituted tetrachlorobiphenyls, the relationships between various substituent parameters and receptor binding constants were examined by means of multiparameter regression analysis.

For each substituent group, a hydrophobic parameter π, an electronic parameter σ, and a hydrogen-bonding accepting parameter HB were determined. The hydrogen-bonding parameter HB is an indicator variable that takes a value of unity for substituents which are hydrogen acceptors but a value of zero for nonhydrogen-bonders. For the electronic effect of 4′-substituents, σ_{para} (Hammett constant) was used. The σ_p values were obtained from the literature.

The hydrophobic parameter π, defined as

$$\pi = \log P_X - \log P_H$$

where P_X and P_H are the partition coefficients of 4′-substituted-2,3,4,5-tetrachlorobiphenyl and 4′-unsubstituted-2,3,4,5-tetrachlorobiphenyl, respectively, in the *n*-octanol/water system, was estimated by means of a recently developed method (*79*). According to this method, π values for the hydrogen-bondable 4′-substituents were calculated by using the equation:

$$\pi_{X/C_6H_4C_6HCl_4} = 0.96\pi_{X/C_6H_5} + 0.19\,\rho_X \qquad (2)$$

where the subscript $X/C_6H_4C_6HCl_4$, denotes π values for the 4′-substituted-2,3,4,5-tetrachlorobiphenyls and the subscript X/C_6H_5 refers to π values for monosubstituted benzenes. As Eq. (2) illustrates, hydrophobic substituent parameters (π) were derived from π values for monosubstituted benzenes. This derivation is valid because 4′-substituted-2,3,4,5-tetrachlorobiphenyl can be considered as a special case of a *para*-disubstituted benzene in which one of the substituent groups remains constant while the other is variable. The π values for disubstituted benzenes have been calculated using parameters from monosubstituted benzenes (*79*). The factor $0.19\rho_X$, describes the solubility-modifying effect of the electron-withdrawing 2,3,4,5-tetrachlorobiphenyl group on the hydrogen-bonding interaction of each 4′-substituent with solvents. For substituents incapable of hydrogen bonding, such as alkyl and halogen, the ρ value is zero. The electron-withdrawing effect of the 2,3,4,5-tetrachlorobiphenyl group can be expressed by the σ_p value, which was estimated as $\sigma_{p/C_6Cl_5} \times 4/5 = 0.19$. The π_{X/C_6H_5} values were taken from the literature.

For 15 compounds of the present series of *para*-substituted biphenyls, electronic, hydrophobic and hydrogen-bonding substituent constants were examined with respect to the EC_{50} values calculated from competition binding assays (shown in Table 4). Multiple regression analysis of the data led to Eq. (3):

$$\log(1/EC_{50}) = \underset{(0.57)}{1.39\sigma} + \underset{(0.43)}{1.31\pi} + \underset{(0.61)}{1.12HB} + \underset{(0.39)}{4.20} \qquad (3)$$

with $n = 15$, $s = 0.31$, $r = 0.916$, $F = 19.20$ ($\alpha < 1\%$), in which hydrophobic π, electronic σ, and a variable HB for hydrogen bond formation are significant parameters determining the variations in binding constants. The

Table 4. Substituent parameters and analysis of binding constants.

4′-Substituent	π_{X/C_6H_5}[b]	ρ_x[c]	$\pi_{X/C_6H_4C_6HCl_4}$	σ	HB	log(1/EC_{50}) Obs	log(1/EC_{50}) Calc[d]	Δ
H	0	0	0	0	0	3.85	4.20	–0.35
OH	–0.67	0.94	–0.46	–0.37	1	4.05	4.20	–0.15
CH_3	0.56		0.54	–0.17	0	4.51	4.67	–0.16
F	0.14	0	0.13	0.06	0	4.60	4.45	0.15
OCH_3	–0.02	0.27	0.03	–0.27	1	4.80	4.98	–0.18
$COCH_3$	–0.55	0.16	–0.50	0.50	1	5.17	5.36	–0.19
CN	–0.57	0	–0.55	0.66	1	5.27	5.52	–0.25
Cl	0.71	0	0.68	0.23	0	5.33	5.41	–0.08
CH_2CH_3	1.02	0	0.98	–0.15	0	5.46	5.27	0.19
Br	0.86	0	0.83	0.23	0	5.60	5.61	–0.01
I	1.12	0	1.08	0.18	0	5.82	5.86	–0.04
$CH(CH_3)_2$	1.53	0	1.47	–0.15	0	5.89	5.92	–0.03
CF_3	0.88	0	0.85	0.54	0	6.43	6.06	0.37
NO_2	–0.28	–0.14	–0.30	0.78	0[e]	4.85	4.89	–0.04
$NHCOCH_3$	–0.97	0.91	–0.76	0	1	5.09	4.32	0.77
C_6H_5	1.96	0	1.88	–0.01	0	5.18[f]	6.65	–1.47
$C(CH_3)_3$	1.98	0	1.90	–0.20	0	5.17[f]	6.41	–1.24
$CH_2)_3CH_3$	2.08	0	1.99	–0.16	0	5.13[f]	6.59	–1.46

[a] Data from Bandiera et al. (*73*) except as noted.
[b] Taken from Hansch and Leo (*80*).
[c] Taken from Fujita (*79*).
[d] Calculated by first correlation equation ($n = 15$).
[e] See text.
[f] Not included in either correlation equation.

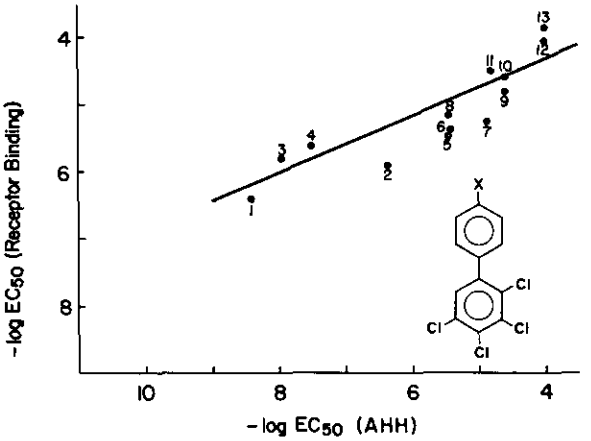

FIGURE 8. Plot of the $-\log EC_{50}$ values of the receptor binding avidities vs. AHH induction potencies for a series of 4'-substituted 2,3,4,5-tetrachlorobiphenyls (Table 3).

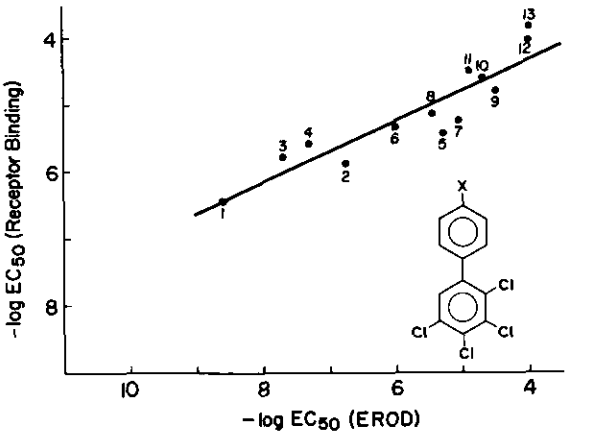

FIGURE 9. Plot of the $-\log EC_{50}$ values of the receptor binding avidities vs. EROD induction potencies for a series of 4'-substituted 2,3,4,5-tetrachlorobiphenyls.

number of compounds is given by n, s is the standard deviation, r is the correlation coefficient, and F is the value of the F-ratio. The figures in parentheses are the 95% confidence intervals. The results of the analysis are shown in Table 4.

The log $(1/EC_{50})$ for the 4'-*N*-acetylamino derivative was most poorly predicted by the Eq. (3). For the 4'-nitro compound, the HB value was taken as zero although the nitro group is capable of acting as a hydrogen acceptor. With an HB value of unity, its affinity was predicted to be about ten times higher than the observed value. The hydrogen-bonding interaction may not be insignificant for this compound although the mechanism is not clear.

The analysis was repeated with exclusion of these two outliers, to yield Eq. (4):

$$\log(1/EC_{50}) = \underset{(0.37)}{1.53\sigma} + \underset{(0.30)}{1.47\pi} + \underset{(0.41)}{1.09HB} + \underset{(0.26)}{4.08} \quad (4)$$

with $n = 12$, $s = 0.18$, $r = 0.978$, $F = 65.90$ ($\alpha < 0.01\%$). Although the quality of the correlation is much improved, the inferences drawn from the correlation are not significantly changed.

The 4'-*n*-butyl, 4'-*tert*-butyl and 4'-phenyl compounds were not included in these correlations. Their $\log(1/EC_{50})$ values were much lower than those predicted by the two correlation equations. The van der Waals volumes of these three substituents were among the highest, being 41.8 cm^3/mole for the *n*-butyl/ and *tert*-butyl groups and 45.8 cm^3/mole for phenyl. The value for other substituents used here was less than 35 cm^3/mole (i.e., 34.12 cm^3/mole for isopropyl and 33.23 cm^3/mole for *N*-acetylamino). Although the use of various types of steric parameters did not produce meaningful correlations, there seems to be a certain limiting size in the receptor binding site(s) to accommodate the biphenyl derivatives.

The correlation (developed in the above equations) means that both increasing substituent electron withdrawing activity and hydrophobicity, increased binding avidity of the ligands for the receptor. Hydrogen bond-accepting substituents also favor this binding. The coefficient of the HB term, 1.10, means that the molarity of the hydrogen donor in the receptor region is 12- to 13-fold that of the octanol phase which is used as a model of the hydrophobic receptor binding site (*81*).

The linear relationship between the AHH and EROD induction EC_{50} values can be expressed by Eq. (5):

$$\log \text{RA (EROD)} = \underset{(\pm 0.180)}{0.983} \log \text{RA (AHH)} - \underset{(\pm 0.235)}{0.539} \quad (5)$$

with $n = 14$, $s = 0.391$, $r = 0.960$. where RA is relative activity, n = number of substituents, s = standard deviation, and r = correlation coefficient. Omitting the data for the noninducers (with estimated EC_{50} values for induction), 4'-hydroxy-2,3,4,5-tetrachlorobiphenyl and 2,3,4,5-tetrachlorobiphenyl, Eq. (5) becomes:

$$\log \text{RA (EROD)} = \underset{(\pm 0.125)}{0.942} \log \text{RA (AHH)} - \underset{(\pm 0.176)}{0.434} \quad (6)$$

with $n = 12$, $s = 0.259$, $r = 0.983$.

The linear correspondence between the relative activities of these two induced enzyme activities provides strong evidence that both enzymes are induced via the same mechanism and that the oxidation of both substrates is catalyzed by the same cytochrome P-450 isozymes.

Equation (7) has been developed to relate the receptor binding affinities to the AHH induction potencies of the substituted halogenated biphenyls:

$$\log \text{RA (AHH)} = \underset{(\pm 0.763)}{2.132} \log (1/EC_{50}) + \underset{(\pm 5.349)}{5.659}\,\Delta B_5 - \underset{(\pm 1.802)}{2.042}\,(\Delta B_5)^2 - \underset{(\pm 5.069)}{14.148} \quad (7)$$

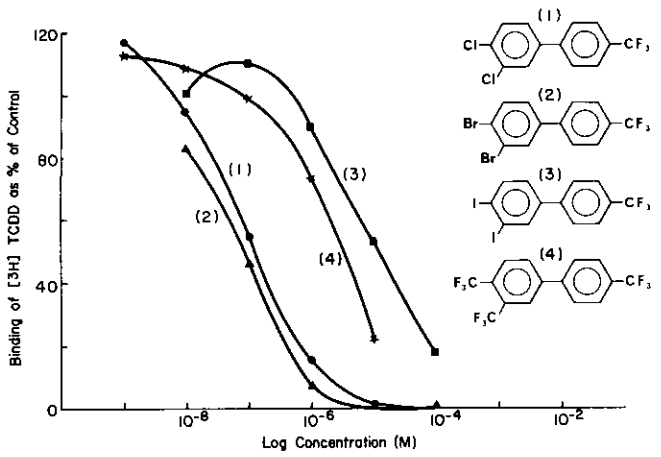

FIGURE 10. Sucrose density gradient analysis after incubation with 10 nM [^{3}H]-2,3,7,8-TCDD with rat cytosolic receptor protein and a series of substituted 4-biphenyl trifluorides.

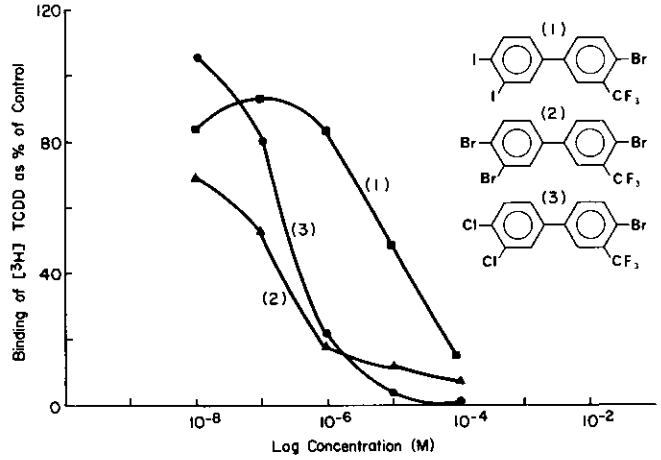

FIGURE 11. Sucrose density gradient analysis after incubation with 10 nM [^{3}H]-2,3,7,8-TCDD with rats cytosolic receptor protein and a series of substituted 4-bromo-3-biphenyltrifluorides.

with $n = 11$, $s = 0.557$, $r = 0.942$. The value ΔB_5 is one of the steric parameters that represent the maximum width of the substituents from the axis connecting the 4′-substituent with the rest of the molecule. ΔB_5 is the value relative to that of hydrogen [$B_5(X) - B_5(H)$]. Although the standard deviation is large, each term is statistically significant (> 95%). The results also show that the AHH induction activity is linearly related to receptor binding (i.e., see Figs. 8 and 9) and the magnitude (2.132) of the coefficient of log ($1/EC_{50}$) indicates that enzyme induction is about two times more sensitive than receptor binding to the structural variations of the 4′-substituents. The AHH induction is also dependent parabolically with the maximum width of the substituent X, the optimum width being 1.4, which is close to the value of the NO_2 and CF_3 substituents. This suggests that the interaction of the ligand:receptor protein complex with the nuclear receptor site is dependent on the conformation of this complex which in turn is governed by the size of the 4′-X substituent.

The importance of the size of the substituents is evident in the receptor binding affinities of a series of halogenated biphenyls which contain two vicinal *meta* and *para* (3′,4′-) substituents in one of the phenyl rings. Figures 10 and 11 summarize the competitive binding affinities of the 3,4-dichloro-, 3,4-dibromo-, and 3,4-diiodo-4′-biphenyltrifluorides and the 4′-bromo-3,4-dichloro-, 3,4,4′-dibromo-, and 4′-bromo-3,4-diiodo-3′-biphenyltrifluorides. The CF_3 group was used in both sets of ligands since this functional group in the 4′-substituted series of compounds facilitated binding to the rat cytosolic receptor protein. For the 4′-substituted halogenated biphenyls, the order of binding avidities for the substituents was I>Br>Cl, and these data could be rationalized by the contributions of their respective electronegativities, lipophilicities and HB capacity. However, for the 3,4-dihalosubstituted biphenyls, the order of binding avidities for both series was Br>Cl>I. This would suggest that steric effects may also play a role in facilitating ligand substituent interactions with the receptor protein and that optimal steric, electronic and lipophilic substituent parameters all contribute to binding affinity of a ligand for the 2,3,7,8-TCDD cytosolic receptor protein. This is also illustrated by the activity of 3,4,4′-tritrifluoromethylbiphenyl in competitively displacing [^{3}H]-2,3,7,8-TCDD from the receptor protein. This ligand contains three substituent groups that are highly electronegative ($\sigma = 0.54$) compared to the Cl, Br and I groups ($\sigma = 0.23$, 0.23, and 0.18, respectively); moreover, there are only small differences in their hydrophobic π values. However, the competitive binding affinity of this ligand for the receptor protein was unexpectedly low.

Current research in our laboratory is focused on *in vitro* QSAR studies with other substituted halogenated biphenyls, dibenzofurans and dibenzo-*p*-dioxins as probes for investigating the structure and function of the 2,3,7,8-TCDD cytosolic receptor protein. This approach is also being used to compare the relative binding affinities of ligands to cytosolic receptor protein from species with different susceptibilities to the effects of toxic halogenated aryl hydrocarbons.

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